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Inhibitor of apoptosis 2 and TAK1-binding protein are components of the *Drosophila* Imd pathway

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The Imd signaling cascade, similar to the mammalian TNF-receptor pathway, controls antimicrobial peptide expression in *Drosophila*. We performed a large-scale RNAi screen to identify novel components of the Imd pathway in *Drosophila* S2 cells. In all, 6713 dsRNAs from an S2 cell-derived cDNA library were analyzed for their effect on *Attacin* promoter activity in response to *Escherichia coli*. We identified seven gene products required for the *Attacin* response *in vitro*, including two novel Imd pathway components: inhibitor of apoptosis 2 (Iap2) and transforming growth factor-activated kinase 1 (TAK1)-binding protein (TAB). Iap2 is required for antimicrobial peptide response also by the fat body *in vivo*. Both these factors function downstream of Imd. Neither TAB nor Iap2 is required for Relish cleavage, but may be involved in Relish nuclear localization *in vitro*, suggesting a novel mode of regulation of the Imd pathway. Our results show that an RNAi-based approach is suitable to identify genes in conserved signaling cascades.

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Introduction

Drosophila melanogaster has developed a highly sophisticated immune defense, which is required for living in a natural environment that is rich in bacteria and fungi. In contrast to mammals, *Drosophila* has no adaptive, that is, antibody-mediated immunity, which makes it a good model for studying the pattern recognition receptors and signaling pathways of innate immunity. In *Drosophila*, there are two major pathways that respond to microbes: the Imd and the Toll pathways. Both of them are strikingly well conserved throughout evolution (Hoffmann *et al*, 1999; Hoffmann 2003; Hultmark 2003). Thus, novel findings from work on *Drosophila* immune response can fuel discoveries in the mammalian systems.

In *Drosophila,* evolutionarily conserved peptidoglycan recognition proteins (PGRPs) are of paramount importance for microbial recognition. Several *Drosophila* PGRPs are necessary for normal resistance to bacteria (Michel *et al*, 2001; Choe *et al*, 2002; Gottar *et al*, 2002; Rämet *et al*, 2002; Takehana *et al*, 2004). Secreted PGRP-SA is essential for induction of immune response genes via the Toll pathway in response to certain Gram-positive bacteria *in vivo* (Michel *et al*, 2001). On the other hand, PGRP-LC is the first component of the Imd pathway (Choe *et al*, 2002; Gottar *et al*, 2002; Rämet *et al*, 2002). It is located on the cell membrane where it appears to act as a pattern recognition receptor for bacteria either alone or together with other PGRPs (Takehana *et al*, 2004). Recently, intracellular domain of PGRP-LC was shown to bind directly to the Imd, which is the next known component downstream of PGRP-LC (Choe *et al*, 2005). Imd contains a death domain with homology to the mammalian receptor-interacting protein 1. The signal is propagated via transforming growth factor-activated kinase 1 (TAK1) to *Drosophila* homologs for IKKγ and IKKα/β (Key and Ird5, respectively) (Rutschmann *et al*, 2000; Lu *et al*, 2001). Whether TAK1 phosphorylates the *Drosophila* IKKs directly is uncertain and the mechanism of TAK1 activation is elusive. TAK1 has also been shown to play a role in the regulation of the c-Jun N-terminal kinase (JNK) pathway (Park *et al*, 2004). Finally, the signal leads to the activation of the *Drosophila* NF-κB homolog Relish, involving its phosphorylation by the IKK complex (Silverman *et al*, 2000) and cleavage by a caspase currently believed to be Dredd (Leulier *et al*, 2000; Stöven *et al*, 2000, 2003), which forms a complex with BG4, a homolog to mammalian Fas-associated death domain protein (FADD; Leulier *et al*, 2002). The phosphorylated and cleaved Relish is then translocated to the nucleus, where it binds to DNA leading to synthesis of antimicrobial peptides.

Genome sequencing projects have created a demand to develop rapid methods to elucidate the biological function of individual genes. RNAi is a tool for screening whole genomes for genes with easily assayed loss-of-function phenotypes. It has been a feasible method for large-scale screening of various phenotypes in *Drosophila* hemocyte-like S2 cells (Rämet *et al*, 2002) including signaling cascades (Lum *et al*, 2003), and a genome-wide RNAi screen for S2 cell viability was published recently (Boutros *et al*, 2004). In our study, we first evaluated the specificity and effectiveness of RNAi treatments in S2 cells, after which we carried out a blind screen for ~6700 dsRNAs to identify novel components of the Imd signaling cascade.

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Results and discussion

RNAi specifically and effectively decreases the expression of the targeted gene in S2 cells

RNAi silences the expression of the targeted gene in S2 cells (Hammond *et al*, 2000). In order to assay the effectiveness of RNAi-based gene silencing in our system, we incubated 2.5×10^6 S2 cells in six-well plates containing 3 ml of medium with or without 10 μg of *CG5210* dsRNA for 48 h. *CG5210* codes for a chitinase-like protein containing a signal sequence. After RNAi, expression levels of more than 13 500 transcripts were measured using oligonucleotide microarrays. Strikingly, only *CG5210* mRNA level was strongly decreased, whereas the rest of the mRNA levels remained unaffected (Figure 1A), showing that RNAi is both effective and highly specific in S2 cells.

RNAi phenotypes are reproducible

RNAi-based *in vitro* screens have been successfully used to identify genes required for various cell-based functions, and we wanted to test whether previously reported RNAi pheno-

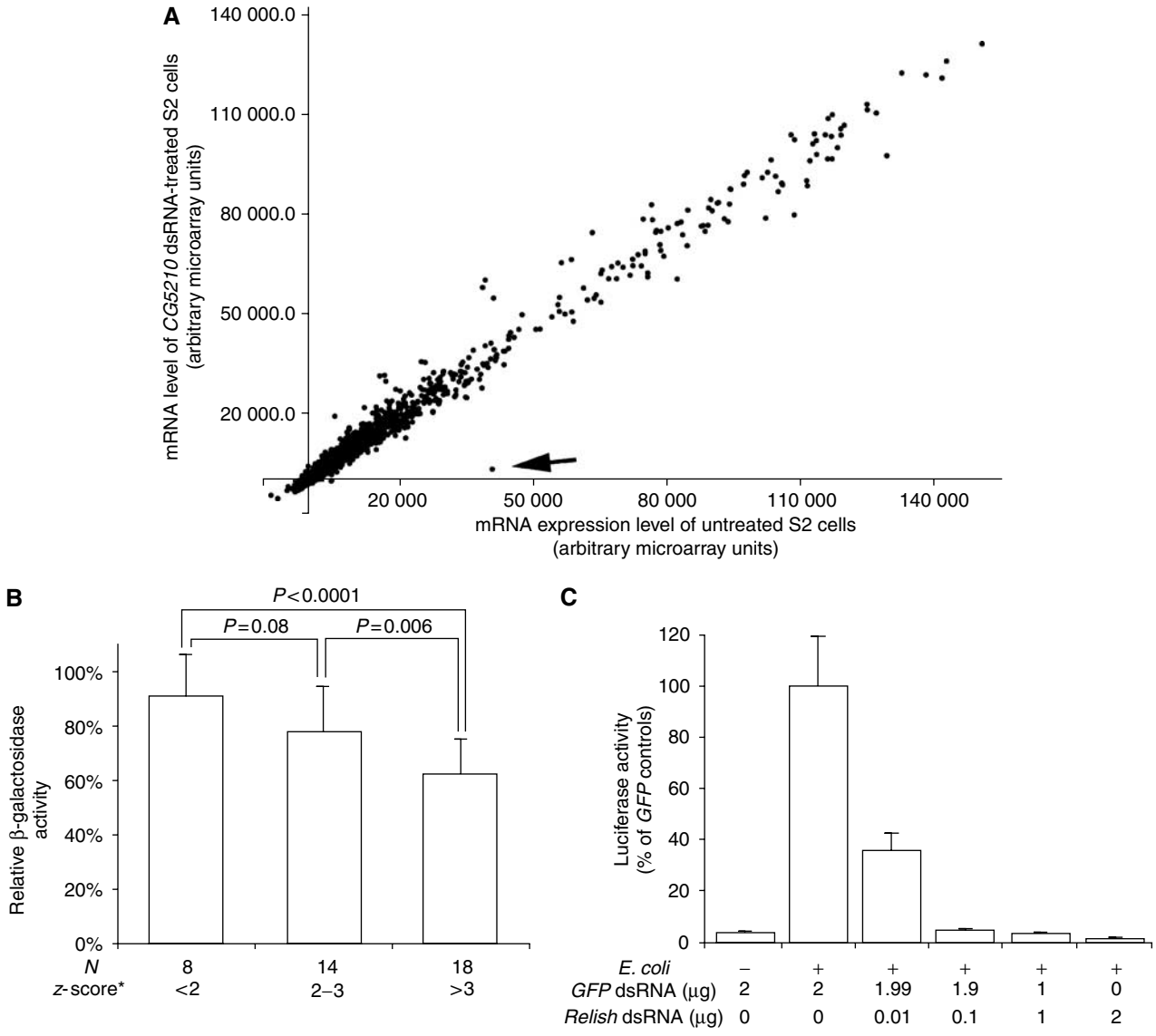

Figure 1 RNAi-based screen to identify components of the Imd signaling cascade in S2 cells. (**A**) RNAi-mediated gene silencing effectively and specifically decreases the mRNA level of the targeted gene in S2 cells. A total of 2.5×10^6 S2 cells in six-well plates containing 3 ml of medium were incubated with or without 10 μg of *CG5210* dsRNA for 48 h. The expression level of more than 13 500 genes was measured using Affymetrix *Drosophila* genechips. Relative expression levels of all genes are shown. Each point represents the average mRNA expression level of three independent *CG5210* RNAi treatments compared pairwise to untreated S2 cells. Arrowhead indicates the dot representing the *CG5210* mRNA level. (**B**) dsRNA treatments targeting ribosomal proteins moderately affect the translation rate of *Act5C* in S2 cells. Expression of 40 genes coding for ribosomal proteins was silenced using RNAi, and translation rate of *Act5C*-β-gal was measured in relation to *GFP* RNAi-treated controls. Gene-specific β-galactosidase activities were thereafter compared to *z*-scores reported by Boutros *et al* (2004). There was a significant correlation between *Act5C*-β-gal activities and *z*-scores (see also Supplementary Table SI). (**C**) *Relish* RNAi blocks the Imd pathway activity in a dose-dependent manner in S2 cells. *Att*-luc plasmid was used to measure the activity of the Imd pathway and *Act5C*-β-gal to normalize the results. A total of 5.0×10^5 S2 cells in 500 μl of medium were treated with the total of 2 μg of the indicated dsRNA(s). *GFP* dsRNA was used as a negative control. Imd pathway was induced with heat-killed *E. coli*, and luciferase and β-galactosidase activities were measured 24 h afterwards. Data are shown as mean ± s.d., $N \geqslant 3$.

types are reproducible in our experimental setting. Recently, Boutros *et al* (2004) showed that nearly all dsRNA treatments targeting genes encoding ribosomal proteins resulted in similar, rather modest phenotypes. To this end, we carried out RNAi experiments to silence the expression of 40 genes coding for ribosomal proteins. We compared the viability of these cells, using a constitutively active promoter *Actin 5C*-driven β-galactosidase reporter (*Act5C*-β-gal) activity, to the results of Boutros *et al* (2004). The result for each individual gene is shown in Supplementary Table SI. There was a significant correlation between our β-galactosidase results and *z*-scores from Boutros *et al* (2004; Figure 1B). *z*-score measures the severity of RNAi phenotype on cell viability; $z=0$ is the average of control dsRNA treatments (no effect on viability), and the higher the score, the less viable the cells are. We observed significantly ($P<0.0001$) lower β-galactosidase values from RNAi treatments targeting ribosomal genes, which had a *z*-score >3 compared to those that had a *z*-score <2 (Figure 1B). Similarly, dsRNA treatments causing *z*-scores 2–3 differed significantly ($P=0.006$) in their respective β-galactosidase values from those having *z*-scores greater than 3 (Figure 1B). Noteworthy, RNAi experiments silencing ribosomal proteins caused relatively mild phenotypes (mild change in β-galactosidase values and *z*-scores; Figure 1B and Boutros *et al*, 2004). These results indicate that RNAi phenotypes can be reproduced in different experimental settings.

Luciferase-based reporter assay to analyze Imd pathway activity in S2 cells

Normal response to most Gram-negative bacteria in *Drosophila* depends on the Imd pathway, which is very similar to the TNF receptor signaling pathway in mammals. In order to determine whether there are still unknown components in the Imd signaling pathway, we carried out a large-scale RNAi-based screen in *Drosophila* S2 cells using a luciferase-reporter-based quantitative assay. The activity of the pathway was assayed using *Attacin*-luciferase (*Att*-luc) reporter (Tauszig *et al*, 2000). Transfection efficiency and cell viability were monitored using *Act5C*-β-gal reporter. The Imd signaling pathway was activated with heat-killed *Escherichia coli*. At first, we tested if dsRNA targeting a known component of the pathway caused a decrease in *Att*-luc activity. As shown in Figure 1C, *Relish* (*Rel*) RNAi blocked the Imd pathway activity in a dose-dependent manner. 10 ng of *Rel* dsRNA per 5.0×10^5 S2 cells in 500 μl of medium reduced the luciferase activity by $>50\%$ and more than 0.1 μg of *Rel* dsRNA blocked the luciferase activity almost completely (Figure 1C). Therefore, RNAi very effectively silences the expression of the targeted gene in our assay, which thus can be used to identify essential components of the Imd pathway.

RNAi analysis of gene products regulating the expression of Attacin via the Imd pathway in S2 cells

We examined 6713 dsRNAs from an S2 cell-derived cDNA library for their effect on the Imd signaling pathway in S2 cells using *Att*-luc reporter as a read-out. Most dsRNA treatments had little or no effect. As shown in Table I, seven genes decreased *Att*-luc activity by $>80\%$ without decreasing *Act5C*-β-gal activity by more than 40%, indicating that viability and the translation machinery were unaffected. These genes included three (*PGRP-LC*, *imd* and *Rel*) out of eight known components of the Imd pathway. *Rel* was identified three times. Novel genes identified were *kayak*, *longitudinals lacking* (*lola*), *inhibitor of apoptosis 2* (*Iap2*) and *CG7417*. The CG7417 protein is a homolog to the mammalian TAK1-binding proteins 2 and 3 (TAB2 and TAB3), hereafter called TAB. RNAi targeting these genes did not affect the *z*-scores in the study of Boutros *et al* (2004), further indicating that these dsRNA treatments have a direct effect on Imd pathway signaling. Interestingly, a dsRNA treatment silencing *Rel*, *TAB*, *PGRP-LC*, *imd* or *lola* also strongly decreased the *Drosomycin* reporter (*Drs*-luc) activity induced by the constitutively active form of Toll (Toll10b; Table I). Therefore, it appears that a low level of *Rel* activity is required also for normal *Drs* response via the Toll pathway in S2 cells.

Kayak is a known component of the JNK signaling pathway; RNAi targeting *kayak* caused an $88\pm7\%$ decrease in *Att*-luc activity (Table I). This is in accordance with our recent results, which indicate that JNK is essential for normal antimicrobial peptide release in S2 cells (Kallio *et al*, 2005). RNAi targeting *lola* caused $87\pm5\%$ decrease in *Att*-luc activity. Lola is a nuclear factor that is required for axon growth in the *Drosophila* embryo (Madden *et al*, 1999) and normal phagocytosis of bacteria in S2 cells (Rämet *et al*, 2002). Lola has not been indicated to play a role in the synthesis of antimicrobial peptides. In our reporter assay, *lola* RNAi decreased *Att*-luc activity slightly less than known

Table I dsRNA treatments specifically decreasing *Att*-luc activity (the Imd pathway)

CG#	Name	*N*	*Att*-luc Mean±s.d. (%)	*Act5C*-β-gal Mean±s.d. (%)	*N*	*Drs*-luc Mean±s.d. (%)	*z*1[a]	*z*2[a]
CG11992	*Rel*	3	1±0	96±22	3	32±1	−0.9	−1.3
CG7417	*TAB*	4	2±1	97±21	6	38±10	0.2	0.4
CG8293	*Iap2*	6	2±1	120±35	3	96±24	0	0.3
CG4432	*PGRP-LC*	4	7±4	99±12	6	35±5	0.1	−0.5
CG5576	*imd*	3	7±1	99±5	5	54±12	−1.6	−0.8
CG15509	*kayak*	2	12±7	142±82	2	71±5	0.1	−0.5
CG12052	*lola*	4	13±5	102±24	4	26±15	0.7	2.1

Act5C-β-gal = *Actin 5C*-driven β-galactosidase reporter; *Att*-luc = *Attacin*-luciferase reporter; *Drs*-luc = *Drosomycin* luciferase reporter; *Rel* = *Relish*; *TAB* = TAK1-binding protein; *Iap2* = *inhibitor of apoptosis 2*; *PGRP* = *peptidoglycan recognition protein*; *lola* = *longitudinals lacking*.
Drs-luc (the Toll pathway) values and *z*-scores (indicator of cell viability; Boutros *et al*, 2004) are also shown.
[a]From Boutros *et al* (2004). *z*1 and *z*2 scores show the effect of indicated RNAi on Kc_{167} cell or $S2R^+$ cell viability, respectively. The higher the score, the less viable are the cells (score 0 represents the *z*-score of control dsRNA-treated cells).

components of the Imd pathway. Of note, RNAi silencing of *lola* also decreased *Drs*-luc activity induced by Toll10b in S2 cells.

In all, 35 dsRNA treatments representing 22 genes caused a greater than three-fold increase in the *Att*-luc activity in response to heat-killed *E. coli* after ecdysone treatment in S2 cells (Supplementary Table SII). These genes could be divided into the following categories based on the putative function of their encoding protein: (1) genes involved in microtubule organization or actin cytoskeleton regulation (*par-1*, *Rab-protein 11*, *multiple ankyrin repeats single KH domain [mask]*, *α-Tubulin at 84B*, *CG6509* and *PDGF- and VEGF receptor related [Pvr]*); (2) helicases and other genes involved in DNA replication (*Helicase 89B*, *Rm62*, *kismet*, *mutagen-sensitive 209* and *double parked*); (3) signaling molecules (*daughter of sevenless*, *CG32782* and *Ecdysone-induced protein 75B*); (4) transcription factors (*E2F transcription factor* and *Zn-finger homeodomain 1*) and (5) uncharacterized genes. Of note, *kismet* was identified eight times, *Pvr* six times and *E2F transcription factor* twice in total in our screen. The mechanisms how these genes affect signaling through the Imd pathway remain to be studied. Of note, none of these dsRNA treatments notably induced the Imd pathway without *E. coli* (data not shown).

We identified 124 dsRNA treatments representing 83 genes that decrease *Act5C*-β-gal activity by $>40\%$. These include transcription factors, genes required for DNA replication, cell cycle control, ubiquitination and lipid metabolism, and genes with unknown function. The complete list of these RNAi target genes, including *Act5C*-β-gal values and *z*-scores from Boutros *et al* (2004), is shown in Supplementary Table SIII.

Iap2 and TAB are novel components of the Imd pathway

Importantly, we identified two novel components of the Imd pathway, Iap2 and TAB, which appear to be absolutely necessary for induction of *Att*-luc activity in S2 cells in response to heat-killed *E. coli*. dsRNA targeting either *Iap2* or *TAB* caused a drastic, $98\pm1\%$ decrease in *Att*-luc activity (Table I). Neither treatment affected S2 cell viability when assessed using either β-galactosidase activity or *z*-score (Table I; Boutros *et al*, 2004). Furthermore, *Iap2* or *TAB* RNAi had no effect on cell growth as determined by cell counts (data not shown), indicating that the result is not due to increased cell death. To verify that our observed phenotypes were caused by decreased expression of *Iap2* and *TAB*, we carried out targeted RNAi with gene-specific primers. As shown in Figure 2A, specific dsRNA treatments targeting either *Iap2* or *TAB* drastically decreased the *Att*-luc activity. *TAB* RNAi also decreased the *Drs* reporter activity via the Toll10b-induced Toll pathway (Table I and Figure 2A). We also examined whether the effect of *Iap2* or *TAB* RNAi was ecdysone dependent. If ecdysone was not used, *Att*-luc induction was clearly ($35\pm2\%$, $N=3$) weaker, but also this induction was blocked by RNAi targeting either *Iap2* or *TAB* (data not shown), indicating an ecdysone-independent mechanism.

To ascertain that our results were not due to an artifact related to the use of a reporter construct, we analyzed the expression level of *Cecropin A1* (*CecA1*), another well-characterized antimicrobial gene regulated by the Imd pathway, by semiquantitative RT–PCR. As shown in Figure 2B, a 6-h exposure to heat-killed *E. coli* increased the mRNA level of *CecA1*. This increase could be blocked entirely by RNAi targeting either *Rel* or *TAB*. In addition, induction was reduced by RNAi targeting *Iap2*. Corresponding results were obtained also for *Att D* and *Diptericin* (*Dpt*). dsRNA treatments targeting either *Rel* or *TAB* totally blocked the induction, while the effect of *Iap2* RNAi was somewhat more moderate (data not shown).

To investigate whether these *in vitro* findings are of *in vivo* relevance, we used the inducible expression of *Iap2* dsRNA in *Drosophila in vivo*. The *UAS/GAL4* binary system to drive expression of dsRNA in a defined tissue has been previously used to block the expression of defined genes (Kennerdell and Carthew, 2000; Leulier *et al*, 2002). To this end, we generated transgenic flies carrying the *UAS-Iap2-IR*. This construct has two 500 bp long inverted repeats (IR) of the gene, separated by an unrelated DNA sequence that acts as a spacer, to give a hairpin-loop-shaped RNA. These transgenic flies were crossed to flies carrying various *GAL4* drivers in order to activate transcription of the hairpin-encoding transgene in the progeny. Iap2 has been shown to be required for the regulation of apoptosis in *Drosophila* (F Leulier, personal communication, 2005), and overexpression of *UAS-Iap2-IR* with the ubiquitous and strong *daughterless-GAL4* (*da-GAL4*) driver lead to lethality at the pupal stage. To address the role of Iap2 in antimicrobial gene expression, we expressed the *UAS-Iap2-IR* transgene using the *C564-GAL4* driver that expresses *GAL4* in the adult fat body. Flies were kept at 25°C to avoid the induction of apoptosis in the fat body. Flies that express *Iap2-IR* ubiquitously through *C564* showed no detectable defects. However, the expression of the antibacterial peptide gene *Dpt* was strongly reduced after infection with the Gram-negative bacteria *Erwinia carotovora* (Figure 2C). This phenotype was similar, although weaker, than those generated by *BG4-IR* RNAi. Importantly, the expression of *Drs* remained inducible in *Iap2-IR*; *C564* flies, indicating that Iap2 did not block the Toll pathway and that the fat body remained functional.

Iap2 and TAB are both located downstream of Imd

To map the locations of Iap2 and TAB in the Imd signaling cascade, we overexpressed known components of the cascade including a constitutively active form of Relish (Rel ΔS29–S45), wild-type Relish or wild-type Imd (Figure 3A). All these caused an activation of *Att* expression in S2 cells. *Att* induction caused by expression of either Relish construct could not be blocked by RNAi targeting either *imd*, *TAB* or *Iap2*, indicating that both TAB and Iap2 are located upstream of Relish (Figure 3A). On the other hand, *Att* induction caused by overexpression of Imd was blocked by RNAi targeting either *Rel*, *imd*, *TAB* or *Iap2*, indicating that both TAB and Iap2 lie downstream of Imd in the hemocyte-like S2 cells.

To assess whether Iap2 is located downstream of Imd in the fat body *in vivo*, we overexpressed the *UAS-imd* construct with a *heat-shock-GAL4* (*hs-GAL4*) driver, which induces expression of the *Dpt* gene in the absence of infection. Although there is some constitutive *Dpt* expression in these flies, the level of *Dpt* increases after heat shock. Using these flies, *Dpt* expression was reduced by coexpression of *UAS-Iap2-IR* by $44\pm8\%$ ($N=2$) in these flies. Total RNA was extracted from unchallenged adult flies, collected 6 or 16 h after a heat shock (37°C, 1 h) and RT–PCR analysis was used

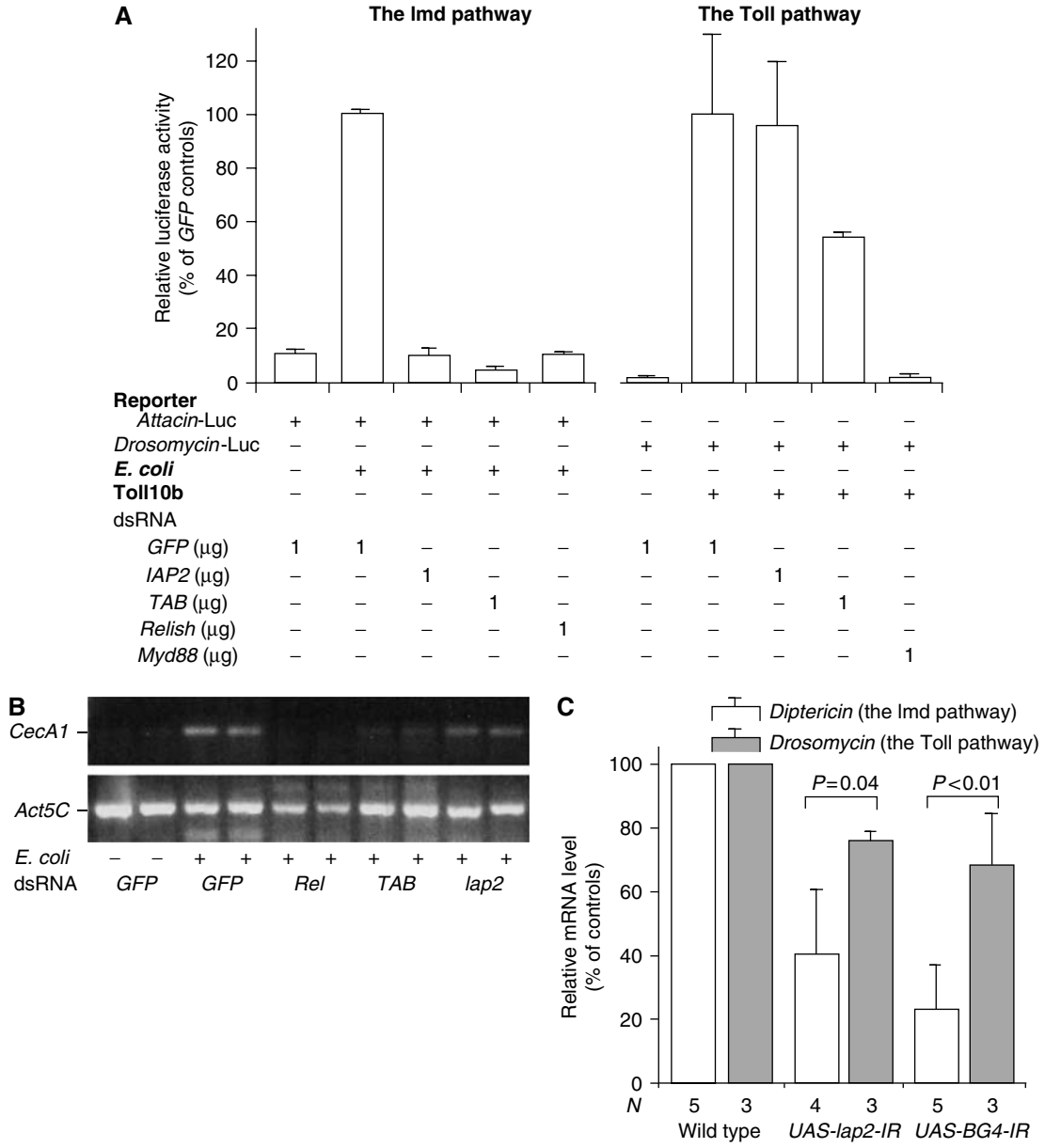

Figure 2 Targeted RNAi of *Iap2* or *TAB* reduces the Imd pathway activity. (**A**) The effect of targeted *Iap2* or *TAB* RNAi on Imd and Toll pathways. S2 cells were transfected either with *Att*-luc (the Imd pathway) or with *Drs*-luc reporter plasmid together with Toll10b (the Toll pathway). *Act5C*-β-gal reporter was included to control transfection efficiency and viability. dsRNA (2 μg) was added directly to transfection medium. For Imd pathway, the transfected cells were incubated with ecdysone (day 3) prior to the addition of heat-killed *E. coli* (day 4), after which the *Att*-luc activity was measured on day 5. The *Drs*-luc activity (Toll pathway) was measured on day 4 after transfection. Data are shown as mean ± s.d., $N \geqslant 3$. (**B**) *CecA1* induction is inhibited by dsRNA treatments targeting either *TAB* or *Iap2*. S2 cells were treated with 20 μg of indicated dsRNA and ecdysone was added 48 h later. After 24 h, the cells were exposed to heat-killed *E. coli* for 6 h, after which the total RNA was extracted. RT–PCR analysis was used to monitor the expression level of *CecA1*. Each treatment was carried out in duplicate. (**C**) Iap2 regulates the expression of antibacterial peptide gene *Diptericin* in *Drosophila* adults. Quantitative RT–PCR analysis was performed with total RNA extracts from wild-type (*C564/+*) flies and flies overexpressing the *UAS-Iap2-IR* or *UAS-BG4-IR* females with *C564*. *Diptericin* expression was monitored in flies collected 6 h after septic injury with *E. carotovora*, while *Drs* was monitored in flies collected 24 h after septic injury with *M. luteus*. Number of experiments (*N*) is shown.

to monitor the expression level of *Dpt*. This indicates that Iap2 functions, genetically, downstream of Imd in the fat body *in vivo*.

To map the exact location of Iap2 in the Imd signaling cascade, we overexpressed Iap2 in S2 cells, which resulted in a minimal but reproducible induction of *Att* expression (Figure 3B). This induction was completely blocked by dsRNAs targeting the known components of the Imd pathway, except dsRNAs targeting either *imd* or *TAK1*. These results indicate that Iap2 lies downstream of TAK1 in the Imd signaling pathway. To ascertain efficacies of the dsRNA treatments used, we simultaneously measured their effect on *E. coli*-induced *Att* response (Supplementary Figure S1). All of the dsRNA treatments strongly decreased the *Att* response, suggesting that the expression of targeted genes was effectively silenced. Of note, we were unable to stimulate the Imd pathway with the expression vector containing the full-length cDNA of TAB.

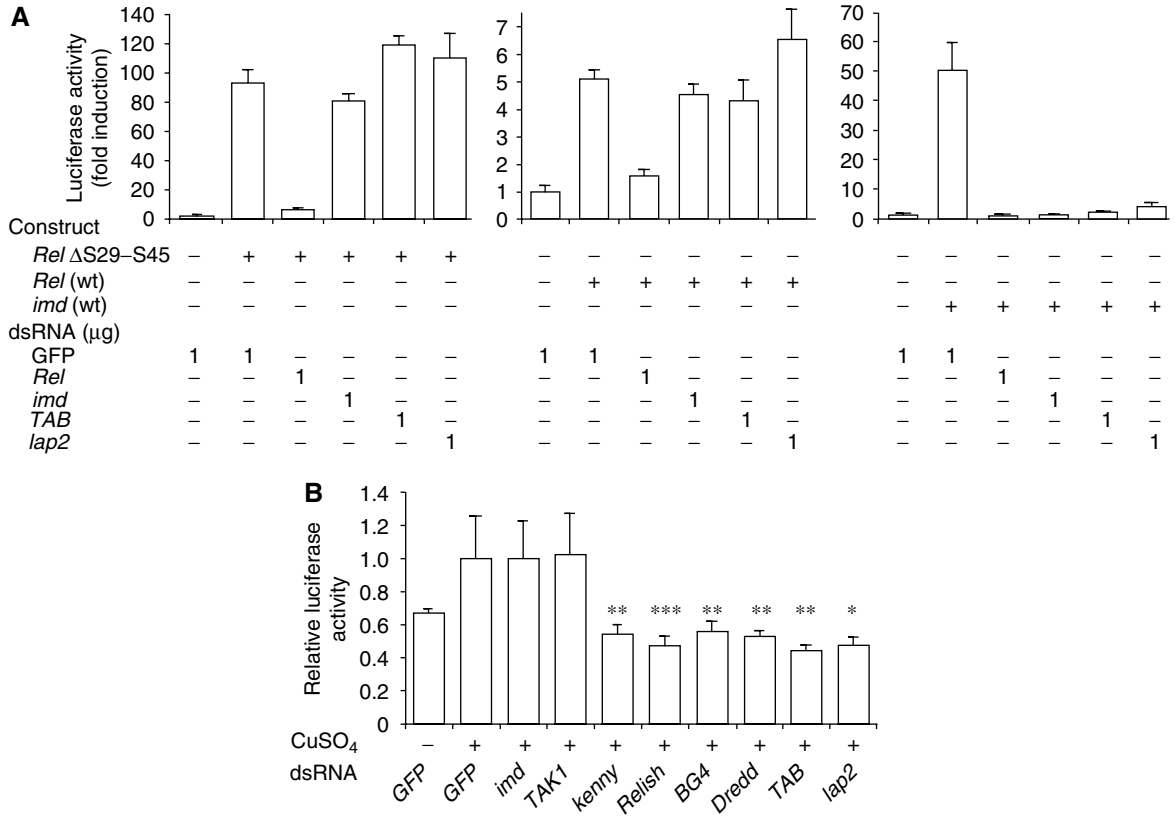

Figure 3 Iap2 and TAB are located below Imd in the Imd signaling cascade. (**A**) Iap2 and TAB are located downstream of Imd. Overexpression of Relish constructs or Imd caused an activation of *Att* response in S2 cells. *Att* induction caused by expression of either Rel ΔS29–S45 or wild-type Relish could be blocked by RNAi targeting *Rel* but not RNAi targeting either *imd*, *TAB* or *Iap2*, indicating that both TAB and Iap2 are located upstream of Relish. *Att* induction caused by overexpression of Imd was blocked by RNAi targeting either *Rel*, *imd*, *TAB* or *Iap2*, indicating that both TAB and Iap2 are downstream of Imd. (**B**) Iap2 lies below TAK1 in the Imd signaling cascade. Overexpression of Iap2 caused a minimal but reproducible induction of *Att* expression, which was blocked by dsRNAs targeting the known components of the Imd pathway except dsRNA targeting either *imd* or *TAK1*. This indicates that Iap2 lies downstream of TAK1 in the Imd signaling pathway. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$, when the *Att* expression levels are compared to *GFP* RNAi controls. Data are shown as mean ± s.d. of three to six independent dsRNA treatments.

Iap2 and TAB appear to be involved in nuclear localization of Relish but are not required for cleavage

Upon Imd pathway activation, the NF-κB homolog Relish becomes phosphorylated by the IKK complex and thereafter cleaved by a caspase putatively thought to be Dredd. Finally, Relish is translocated to the nucleus. To study the role of TAB and Iap2 on Relish cleavage, we stimulated *Drosophila* hemocyte-like mbn-2 and S2 cells with commercial lipopolysaccharide (LPS) known to contain a bacterial component that activates the Imd pathway, followed by Western blotting with Relish antibody (α-C; Stöven *et al*, 2000). As shown in Figure 4A, in unstimulated, *GFP* dsRNA-treated mbn-2 cells, most of Relish is uncleaved (Relish-110 (REL-110)), whereas upon LPS stimulus, Relish cleavage is induced. As expected, in *Dredd* and *key* dsRNA-treated cells Relish cleavage was blocked. Interestingly, *TAB*, *Iap2* or *TAK1* dsRNA did not affect Relish cleavage (Figure 4A). Similar results were obtained also in S2 cells (Figure 4B and data not shown). This points to a novel mechanism of regulation of Relish activity. There was no Relish detected in *Rel* dsRNA-treated cells, indicating that the half-life of REL-49 (C-terminal Relish cleavage product) is less than the duration of the dsRNA treatment. Of note, REL-49 was observed also in all *Dredd* dsRNA-treated cells. It is possible that after RNAi knockdown, there is a small amount of Dredd left, sufficient to cleave REL-110 in unstimulated cells. Alternatively, there is some constitutively cleaved Relish in cell lines and this cleavage is Dredd independent.

To investigate whether Iap2 or TAB play a role in the nuclear localization of the activated Relish protein, dsRNA-treated S2 cells were stained with α-RHD antibody (Stöven *et al*, 2000). In *GFP* dsRNA-treated cells, Relish is translocated into the nucleus upon LPS stimulus (Figure 4C). As expected, there is no nuclear staining of Relish in *Dredd* or *key* dsRNA-treated, LPS-stimulated S2 cells. Importantly, the nuclear translocation of Relish appears to be affected in both *Iap2* and *TAB* dsRNA-treated cells compared to *GFP* dsRNA-treated controls (Figure 4C). This suggests that cleavage of Relish is not sufficient for translocation of Relish to the nucleus but another, yet to be characterized signal that is propagated via Iap2 and TAB is required. Alternatively, different staining pattern could be due to decreased stability of nuclear Relish or slower kinetics. Of note, compared to *key* and *Dredd* dsRNA-treated cells, very faint nuclear staining can be seen in Iap2 dsRNA-treated cells. Surprisingly, Relish nuclear localization was normal in *TAK1* dsRNA-treated cells. This implies a possibility that the role of TAK1 in the Imd pathway signaling is downstream of translocation of Relish into the

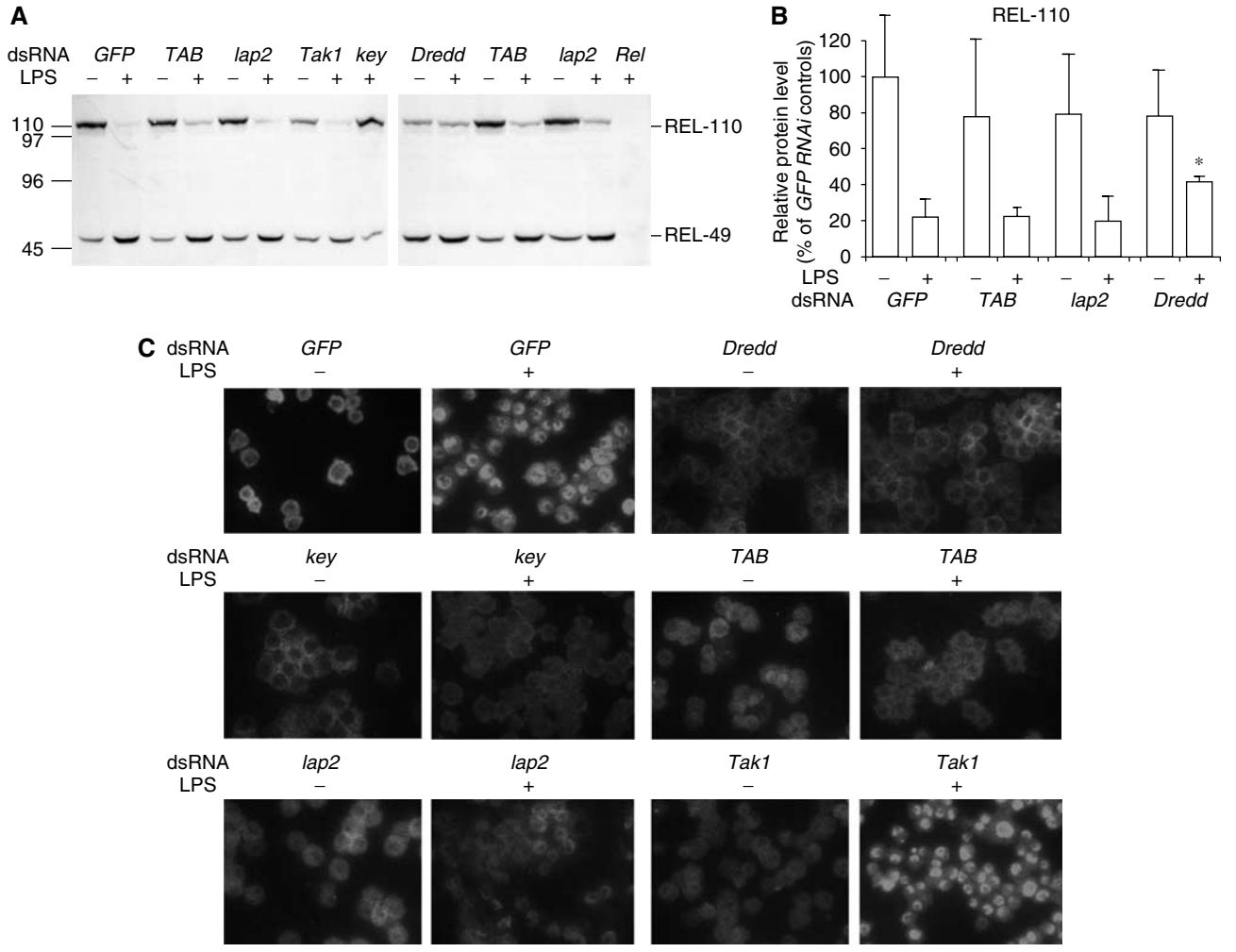

Figure 4 Neither Iap2 nor TAB is needed for Relish cleavage, but both appear to be involved in Relish nuclear localization. (**A**) Iap2 and TAB are not required for Relish cleavage. Western blots of protein extracts (25 μg protein/lane) from LPS-induced mbn-2 cells, using α-C Relish antibody. Mbn-2 cells were treated with 20 μg of indicated dsRNA for 72 h and thereafter incubated for 30 min with LPS. (**B**) Quantification of REL-110 bands from Western blots using Bio-Rad Quantity one software (version 4.5.2.). Mbn-2 or S2 cells were treated with 20 μg of indicated dsRNA for 72 h and thereafter incubated for 30 min with LPS. $^*P = 0.03$; *Dredd* RNAi inhibits LPS induced cleavage of Relish compared to *GFP* RNAi-treated control cells. *Iap2* and *TAB* RNAi treatments have no effect. Data are shown as mean ± s.d. of at least three independent experiments. (**C**) *Iap2* and *TAB* RNAi affect the nuclear localization of Relish. S2 cells were treated with indicated dsRNA for 72 h followed by 10-min exposure to LPS. Thereafter, immunostaining of Relish with α-RHD antibody was performed.

nucleus. Altogether, these result show that the regulation of Relish activity is more complex than previously thought. The involvement of TAB and Iap2 in nuclear localization but not cleavage of Relish indicates a novel mode of regulation in the Imd pathway.

Iap2 and TAB both have mammalian homologs

Iap2 codes for a 498 amino-acid (aa) protein that has three N-terminal BIR (baculovirus IAP repeat) domains and a C-terminal RING-finger (Really Interesting New Gene) domain. *Drosophila* Iap2 is well conserved throughout phylogeny and has high sequence similarity with many mammalian Iap2s, such as human, rat and mouse (*E* values 9×10^{-66}, 2×10^{-66} and 3×10^{-66}, respectively). Interestingly, the CARD (caspase recruitment) domain, identified in apoptotic signaling proteins, is present in the mammalian homologs but missing from *Drosophila*. An alignment highlighting conserved domains is shown in Figure 5A. It has been shown that RING domain containing proteins, including IAPs, bind E2 ubiquitin-conjugating enzymes catalyzing the transfer of ubiquitin from E2 to a substrate, therefore acting as E3 ligases. Ubiquitination can lead to either proteasomal degradation, or, in the case of non-K48-linked polyubiquitination, to multiple outcomes such as activation or relocalization of the substrate protein (Vaux and Silke, 2005). Human c-Iap2 is expressed most strongly in immune tissues including spleen and thymus and has been proposed to associate with TRAFs through its BIR domains (Rothe *et al*, 1995). However, in our luciferase assay, neither *TRAF1* nor *TRAF2* dsRNA treatment reduced the Imd pathway activity, indicating that TRAFs are not essential for Imd pathway activity in *Drosophila* S2 cells.

The *Drosophila TAB* codes for an 831 aa protein that has an N-terminal CUE domain (97–139 aa) and a C-terminal zinc-finger (ZnF) domain (765–789 aa). Only two other *Drosophila* genes code for a CUE domain: *CG2701*, and *CG12024*. Their function is unknown. TAB is the only *Drosophila* protein with both CUE and ZnF domains. These domains are homologous to the respective domains in mammalian TABs (Figure 5B).

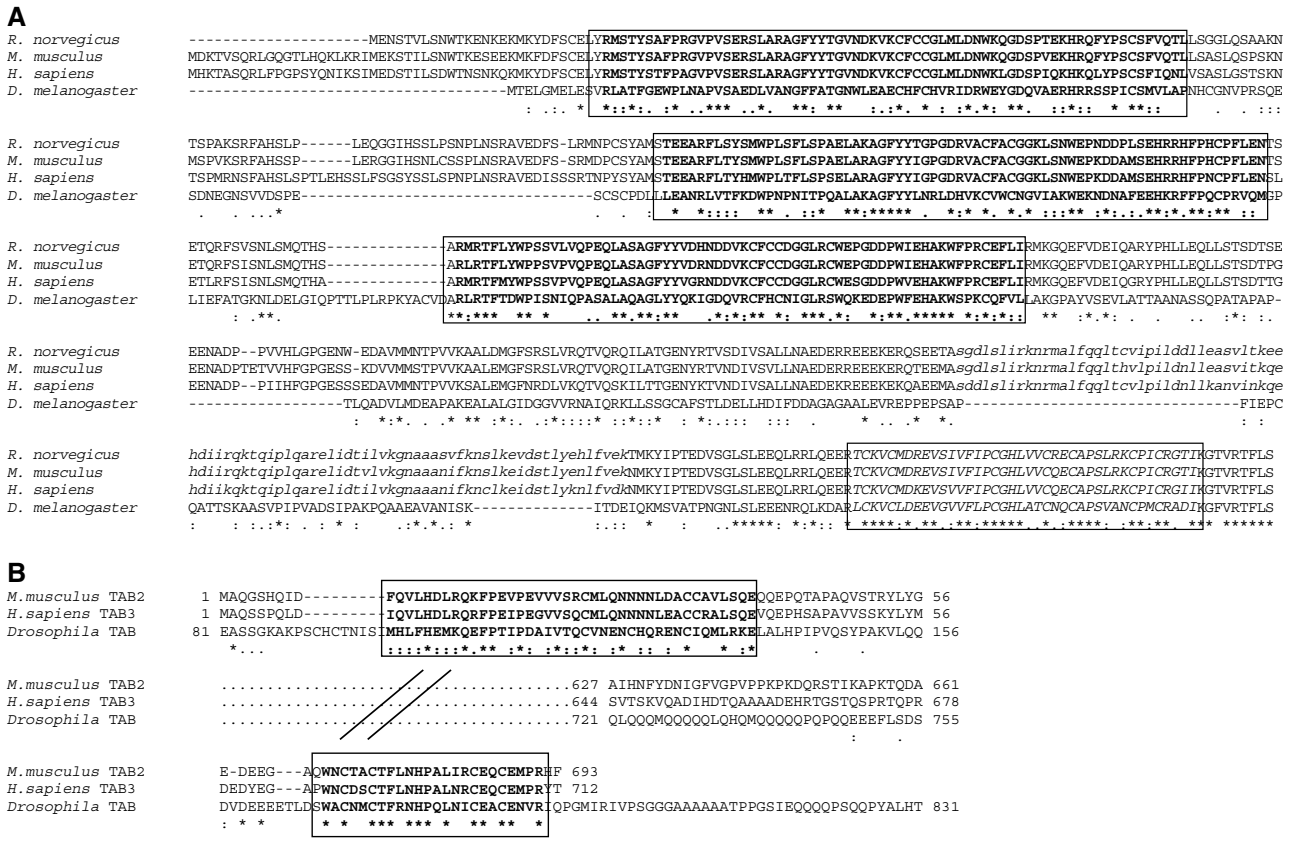

Figure 5 Iap2 and TAB are conserved throughout phylogeny. (**A**) Comparison of the deduced amino-acid sequences of Iap2 homologs from rat (*Rattus norvegicus*, AAH62055.1), mouse (*Mus musculus*, AAC53532.1), human (*Homo sapiens*, NP_001157.1) and *Drosophila melanogaster* (AAF58095.1). The three conserved BIR domains are boxed and shown in bold; CARD domain (missing from *Drosophila*) is shown in lower case and italicized; the RING domain in the C-terminus is boxed and italicized. (**B**) Alignment of *Drosophila* TAB with human TAK1-binding protein 3 (AAQ88279.1) and mouse TAK1-binding protein 2 (NP_619608.1). CUE (97–139) and ZnF (765–789) domains are shown boxed and in bold. The low complexity region (157–720) showing no homology with other TABs is not shown. Alignments were created using ClustalW program.

The CUE domain carries a ubiquitin-binding motif, whereas the ZnF domain has an α-helical coiled-coil region. It has been shown that *Drosophila* TAK1 binds TAB (CG7417) in a two-hybrid protein interaction system (Giot *et al*, 2003). In humans, the C-terminal coiled-coil domain of TAB3 mediates the association with TAK1, and it is also required for stimulation of TAB3 ubiquitination by TRAF6 (Ishitani *et al*, 2003). Apart from these two domains, there is very little sequence similarity, suggesting that these domains are functionally important. Indeed, it has been shown that the ZnF domain and the CUE domain, to a lesser extent, of human TAB2 and TAB3 are important to NF-κB activation (Kanayama *et al*, 2004). Our current hypothesis of signaling via the Imd pathway in *Drosophila* is shown schematically in Figure 6. The exact molecular mechanism by which Iap2 and TAB modulate signaling via the Imd pathway in *Drosophila* remains to be studied.

Conclusion

RNAi has proven to be an efficient method specifically to silence targeted genes and it has been successfully used in large-scale screens of gene function in *Drosophila* cells (Rämet *et al*, 2002; Lum *et al*, 2003; Boutros *et al*, 2004). In this study, we have shown that RNAi is both effective and extremely specific in our experimental setting. Thereafter, we carried out a blind screen for ∼6700 dsRNAs. We identified altogether 29 gene products that played a role in the Imd pathway signaling in S2 cells. Several housekeeping genes that are required for normal *Act5C* promoter-driven β-galactosidase expression were also identified. We found three out of eight previously known components of the Imd pathway. We were unable to find five known components from our screen including *TAK1*, *kenny*, *ird5*, *BG4* and *Dredd*. Of note, targeted RNAi treatments silencing these genes resulted in strongly reduced *Att* promoter activity in our experimental setting. Therefore, we believe that those genes were not present in our dsRNA collection.

We observed very few dsRNA treatments that reduced *Att*-luc activity by more than 80% without significantly affecting the viability of S2 cells as assayed by *Act5C*-β-gal activity. This differs from the results reported recently by Foley and O'Farrell (2004), who found 49 genes from their RNAi screen to be required for normal *Dpt* response. Fewer genes identified by us could simply reflect the smaller number of dsRNA treatments analyzed in our study. However, if we had not used an internal control (namely *Act5C*-β-gal expression), to exclude genes that have a more generalized effect on S2 cell homeostasis, we would have had a much greater number of genes putatively affecting the *Att* expression. Our quantitative and reproducible assay let us use a strict threshold that

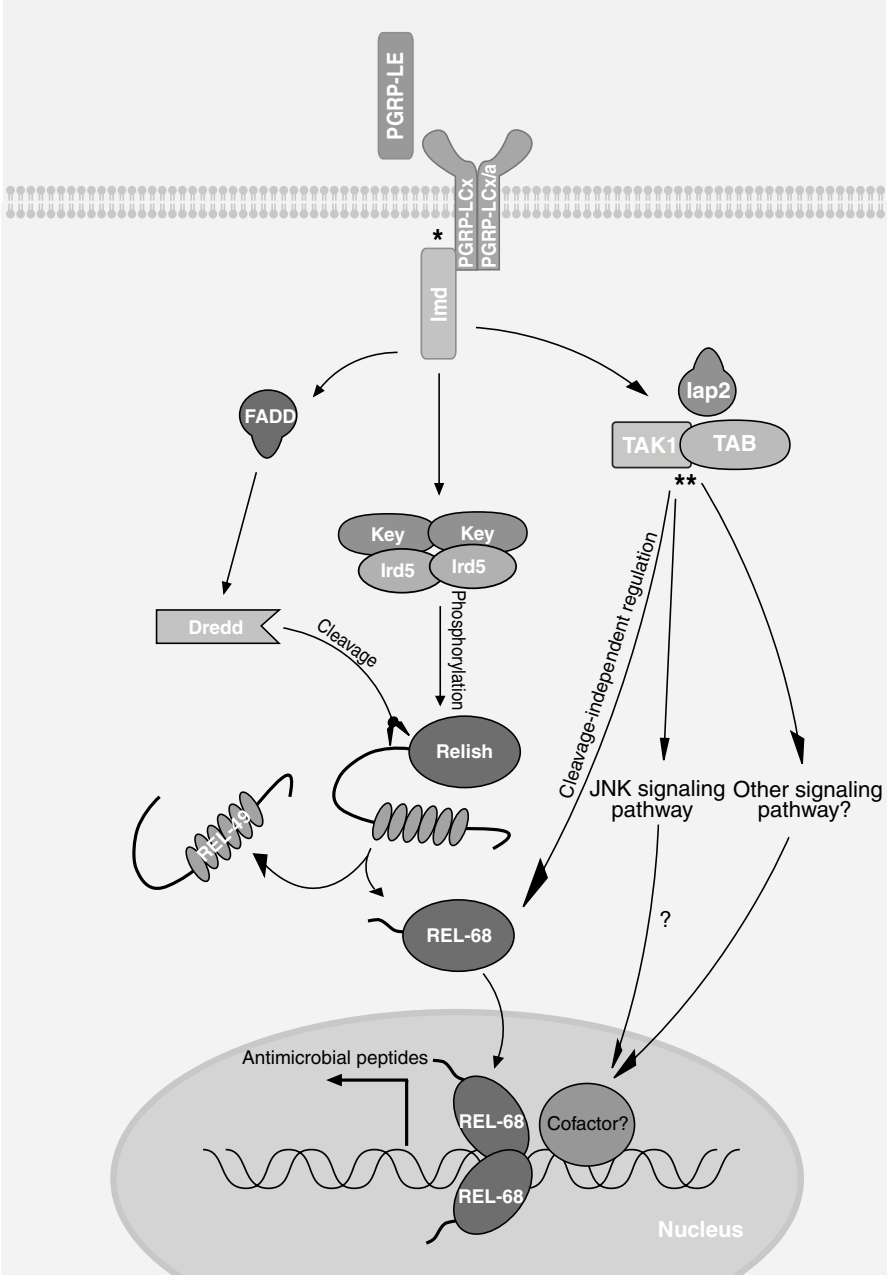

Figure 6 Schematic representation of the Imd signaling pathway in *Drosophila* S2 cells. The asterisk (*) represents a physical interaction of PGRP-LC and Imd detected by immunoprecipitation assay (Choe *et al*, 2005). Two asterisks (**) represents the interaction of TAB and TAK1 detected in a yeast two-hybrid screen (Giot *et al*, 2003). As neither TAB, TAK1 nor Iap2 dsRNA treatment affected Relish cleavage, we conclude that these factors affect antimicrobial peptide release via a cleavage independent mechanism. We speculate that the mechanism may involve Relish nuclear transportation, JNK pathway activation or possibly the activation of another yet unidentified transcriptional cofactor for Relish (question marks).

allowed us to identify true components of the Imd pathway. There may also be differences in our S2 cells and those used by Foley and O'Farrell (2004) that explain the different set of genes identified in these two studies. This is supported by the notion that targeted dsRNA treatments silencing either *Act5C*, *SCAR* or *Dnr1*, reported to activate the Imd pathway by Foley and O'Farrell (2004), did not cause induction of *Att* promoter in our assay. The effectiveness of the *SCAR* and *Act5C* dsRNAs was evaluated by measuring the rate of phagocytosis of *E. coli* and was found to be reduced as expected (Rämet *et al*, 2002; Pearson *et al*, 2003). Of note, our results are in agreement with current results from the laboratory of M Boutros (Gesellchen *et al*, *EMBO Reports*, in press) who also screened for new components of the Imd pathway in *Drosophila* S2 cells.

Importantly, we were able to identify two novel components of the Imd signaling cascade: Iap2 and TAB. Both of these have mammalian homologs, further indicating high conservation of this signaling cascade. TAB is an 831 aa protein that has conserved CUE and ZnF domains. As in mammals, it is plausible that TAB regulates TAK1 activity also in *Drosophila*, as TAB was the only protein to bind TAK1 in a two-hybrid protein interaction system (Giot *et al*, 2003). We have shown that both Iap2 and TAB are located

downstream of Imd. Interestingly, Relish is cleaved appropriately without Iap2 or TAB, but there appears to be an effect to the transportation of Relish to the nucleus. Therefore, we speculate that there is another, previously unidentified level of regulation needed for Relish activation. Since the RING domain-containing Iap2 is a putative E3 ligase, we hypothesize that this regulation could involve ubiquitination of Relish—or another protein regulating the activity of Relish—by Iap2. Possible interaction of Iap2 with the other Imd pathway components remains to be studied.

Surprisingly, Relish nuclear localization was normal in *TAK1* dsRNA-treated cells. This implies a possibility that the role of TAK1 in the Imd pathway signaling is downstream of translocation of Relish into the nucleus. Our results are in line with recent results from J Delaney *et al* (in preparation), which indicate that Relish activation is intact in *TAK1* mutant flies. Therefore, TAK1 may control the activity of another transcription factor—possibly via the JNK pathway—required for normal antimicrobial peptide response in *Drosophila*. This is in line with identification of Kayak as an important factor for *Att* response in this study and with our earlier results indicating that the JNK pathway is required for normal *Att* response in S2 cells (Kallio *et al*, 2005). Of note, the effect of dsRNA treatments targeting JNK pathway components is more modest compared to *TAK1* RNAi in our experimental setting. This is in line with the earlier results of Silverman *et al* (2003), who showed that in S2* cells *TAK1* RNAi totally blocks *Dpt*, *Cecropin* and *Att* response to LPS, whereas RNAi targeting JNK pathway components *hemipterous* and *basket* have more moderate effect. Nevertheless, the regulatory interplay that has been detected between the Imd and the JNK pathway in the *Drosophila* innate immune response (Boutros *et al*, 2002; Park *et al*, 2004) is likely to attract more attention in the future.

This present study underlines the convenience of RNAi-based screening in S2 cells. Importantly, we identified two novel components of the Imd pathway. The exact roles Iap2 and TAB play in the activation of Relish remain to be solved. In addition, our findings will likely focus attention to investigate the importance of Iap2 in mammalian TNF receptor signaling. This methodology can be readily applied to study other conserved signaling cascades.

Materials and methods

Synthesis of dsRNAs

A cDNA library derived from S2 cells cloned into pcDNAI plasmids (Invitrogen) (Pearson *et al*, 1995) was used as a source for templates for dsRNA synthesis. First, competent MC1061/P3 *E. coli* (Invitrogen) transformed with the cDNA library were plated onto ampicillin (20 μg/ml) and tetracycline (8 μg/ml) containing plates. Then, plasmids from individual colonies were isolated and the random gene products from the cDNA library were amplified by two-stage PCR. The following outer primers were used:

5′-CAAGCTTGGTACCGAGCTC-3′ and
5′-CTGCTCCCATTCATCAGTTC-3′.

In the nested PCR, the binding sites for T7 RNA polymerase were included in both ends of PCR primers. The following nested primers were used:

5′-TAATACGACTCACTATAGGGCGGATCCACTAGTAACGG-3′ and
5′-TAATACGACTCACTATAGGGAGGTGACACTATAGAATAGG-3′.

Control dsRNAs were produced using targeted RNAi primers (see Supplementary Table SIV). cDNA from S2 cells was used as template essentially as described earlier (Rämet *et al*, 2001). Negative control *GFP* dsRNA was produced using pMT/BiP/V5-His/GFP plasmid (Invitrogen) as template. dsRNAs were produced from the nested PCR templates by *in vitro* transcription using the T7 MegaScript RNA polymerase (Ambion) according to the manufacturer's instructions. dsRNA for *CG5210* was created by cloning a PCR fragment of 698 bp into pCR-Blunt II-TOPO (InVitrogen), using the following primers: CCCAAGAATAAGCCGAAGAA and CTCACC CAGATGCCATTGT. Purified plasmid DNA was cut with *Hind*III or *Xba*I and used to template *in vitro* RNA synthesis with T7 or SP6 RNA polymerase (Ambion), respectively.

Cell culture, dsRNA treatments and luciferase reporter assay

S2 cells were cultured in Schneider medium (Sigma) + 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 25°C. Mbn-2 cells were cultured in Schneider medium + 10% FBS and 10 mM L-glutamine (Sigma) at 25°C. Luciferase reporter assays were performed essentially as described earlier (Rämet *et al*, 2001; Kallio *et al*, 2005). Briefly, for Imd pathway, S2 cells were transfected with *Attacin*-luc reporter (Tauszig *et al*, 2000), *Actin 5C*-β-galactosidase and dsRNAs using Fugene® transfection reagent (Roche) according to the manufacturer's instructions. Ecdysone (1 μM, Sigma; Dimarcq *et al*, 1997) was added 48 h after transfection and the pathway induced with heat-killed *E. coli* 60 h after transfection. At 90 h after transfection, S2 cells were harvested by centrifugation and lysed in Passive Lysis Buffer (Promega). For Toll pathway, S2 cells were transfected with Toll10b, a constitutively active form of Toll receptor (Rosetto *et al*, 1995), *Drosomycin*-luciferase reporter, *Actin 5C*-β-galactosidase and dsRNAs as above. Luciferase and β-galactosidase activities were measured using standard procedures.

Data analysis

The ratio of luciferase activity and β-galactosidase activity per sample was calculated and the values from each series were normalized by setting the value of the lysate from S2 cells treated with *GFP* dsRNA to one. β-galactosidase activity was normalized by dividing the values by the average of all β-galactosidase measurements. Tested dsRNA treatments were sorted into three groups: (1) dsRNAs decreasing luciferase activity but not significantly affecting β-galactosidase activity (Luc/β-gal $\leqslant 0.2$ and β-gal/β-gal $\geqslant 0.6$), (2) dsRNAs increasing luciferase activity but not significantly affecting β-galactosidase activity (Luc/β-gal $\geqslant 3.0$ and β-gal/β-gal $\geqslant 0.6$) and (3) dsRNAs decreasing β-galactosidase activity (β-gal/β-gal $\leqslant 0.6$). Statistical analysis of results was carried out using one-way ANOVA. $P < 0.05$ was considered to be significant.

Genome-wide analysis of mRNA levels using oligonucleotide microarrays

Total RNA was extracted from 1.0×10^7 S2 cells using RNeasy Mini Kit (Qiagen, CA, USA). Gene expression analysis was performed using the Affymetrix (Santa Clara) *Drosophila* Genechips according to the standard Affymetrix GeneChip protocol as outlined in the GeneChip Expression Analysis Technical Manual by Affymetrix (2001). Gene expression levels of three *CG5210* RNAi-treated S2 cells were compared pairwise to untreated S2 cells.

Sequencing

DNA was purified from PCR reactions with GENECLEAN Turbo Kit (QBiogene) or from an agarose gel with QIAquick Gel Extraction Kit (Qiagen). The samples were prepared for the sequencing with ABI BigDye terminators (Applied Biosystems), essentially as recommended by the manufacturer. Excess dye-labeled terminators and buffers were removed by ethanol/EDTA precipitation, and DNA sequencing was performed with an ABI 3100 automated sequencer (Applied Biosystems).

RT–PCR for assaying CecA1 mRNA level

A total of 1.5×10^6 S2 cells were seeded onto six-well plates and treated with 20 μg of sample or control dsRNA. Ecdysone (1 μM, Sigma) was added 48 h after dsRNA treatment. Antimicrobial peptide release was induced by heat-killed *E. coli* treatment (4 h), after which the total RNA was extracted, 72 h after the dsRNA treatment, with TRIzol® Reagent (Invitrogen) according to manufacturer's instructions. cDNA synthesis was carried out as follows: Template RNA, Oligo(dT)-primer (20 ng/μl) and M-MuLV-buffer were mixed, incubated at 70°C for 5 min and cooled. dNTP (final conc. 1 mM), RNAse inhibitor (20 U, Fermentas) and RNAse free

H_2O were added and the mixture incubated at 25°C for 5 min, after which the M-MuLV reverse transcriptase enzyme (20 U, Fermentas) was added and the mixture incubated for 60 min at 37°C. Finally, the mixture was denatured at 95°C for 5 min, cooled and stored at −70°C. PCR reactions for *CecA1* and *Act5C* were carried out according to manufacturer's instructions using the synthesized cDNAs as templates. Primers and sizes of PCR products are listed in Supplementary Table SIII.

Fly stocks

RNAi transgenic fly lines of *Iap2* were obtained using the inducible RNAi method. A cDNA fragment corresponding to the first 500 bp of the coding sequence was amplified by PCR, and inserted as an IR in a modified transformation vector (pUAST-R57) possessing an IR formation site consisting of paired *Kpn*I–*Cpo*I and *Xba*I–*Sfi*I restriction sites. pUAST-R57 has a 282-bp-long genome fragment of the *Drosophila Ret* oncogene, in which introns 5 and 6 are contained, between two IR fragments to enhance RNAi effect (Kalidas and Smith, 2002). The IR was constructed in a head-to-head orientation by using a combination of tag sequences of PCR primers and restriction sites on the vector. Detailed cloning procedures will be described elsewhere (R Ueda *et al*, manuscript in preparation). Transformation of *Drosophila* embryos was carried out in w^{1118} fly stock. Each experiment was repeated using two independent *UAS-RNAi* insertions. The *BG4-IR* (*dFADD-IR*) fly line has been described previously (Leulier *et al*, 2002). In this study, we used adult flies carrying one copy of the *UAS-RNAi* construct combined with one copy of the *GAL4* driver. The *C564-GAL4* driver expresses strongly GAL4 in adult hemocytes and fat body.

Infection

Bacterial infections were performed by pricking adults with a thin needle dipped into a concentrated culture of bacteria. Details on infection are described elsewhere (Tzou *et al*, 2002; Pili-Floury *et al*, 2004).

Quantitative real-time PCR

For *Drs* and *Dpt* mRNA quantification from whole animals, RNA was extracted using RNA TRIzol® Reagent and cDNAs were synthesized using SuperScript II (Invitrogen). Primer pairs used are listed in Supplementary Table SIV. SYBR Green analysis was performed on a Lightcycler (Roche). All samples were analyzed in duplicate and the amount of mRNA detected was normalized to control *RP49* mRNA values. Normalized data were used to quantify the relative levels of a given mRNA as described in Pili-Floury *et al* (2004).

Overexpression of Iap2 in S2 cells

Full-length *Iap2* cDNA (LD34777), obtained from *Drosophila* Genomics Resource Center (DGRC; Indiana, USA), was subcloned into *Eco*RI and *Xho*I sites of the *Drosophila* expression vector pMT/BiP/V5/HisA. *E. coli* TOP10 cells were transformed with the construct and grown under ampicillin selection. After identification of the right clone by *Eco*RI and *Xho*I digestion, the plasmid was extracted and used (0.5 μg/well) in transfection together with *Att*-luc, *Act5C*-β-gal and dsRNAs as described previously. Overexpression of the Iap2 protein was induced by adding $CuSO_4$ (500 μM) 48 h after transfection. Luciferase and β-galactosidase activities were measured 48 h later and data analysis was carried out as described previously.

Immunohistochemistry

Cells were seeded on a 24-well plate and treated with dsRNAs (4 μg/well, by soaking) for 72 h. Relish cleavage was induced with LPS (final concentration of 10 μg/ml) for 10 min, after which the cells were spun onto glass slides, fixed and labeled with α-RHD antibody as described previously (Stöven *et al*, 2000).

Protein extraction, SDS–PAGE and Western blotting

Cells were seeded onto six-well plates and treated with control or experimental dsRNAs (20 μg/well) for 72 h. Relish cleavage was induced by incubating the cells with LPS (10 μg/ml) for 30 min. Cells were placed on ice and protein lysates prepared as described previously (Stöven *et al*, 2003). In all, 25 μg of protein per sample was used for SDS–PAGE. Electrophoresis and immunoblotting with α-C antibody was carried out as described previously (Stöven *et al*, 2000).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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